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(54)Title: **RECOMBINANT SENDAI VIRUS**

(54)発明の名称 組換え体センダイウイルス

(57) Abstract

A method for reconstituting Sendai virions which comprises introducing the genome of Sendai virus into a host wherein all of the early replication genes have been expressed. This method enables gene manipulations of Sendai virus and thus makes it possible to utilize Sendai virus efficiently as a vector.

図 1

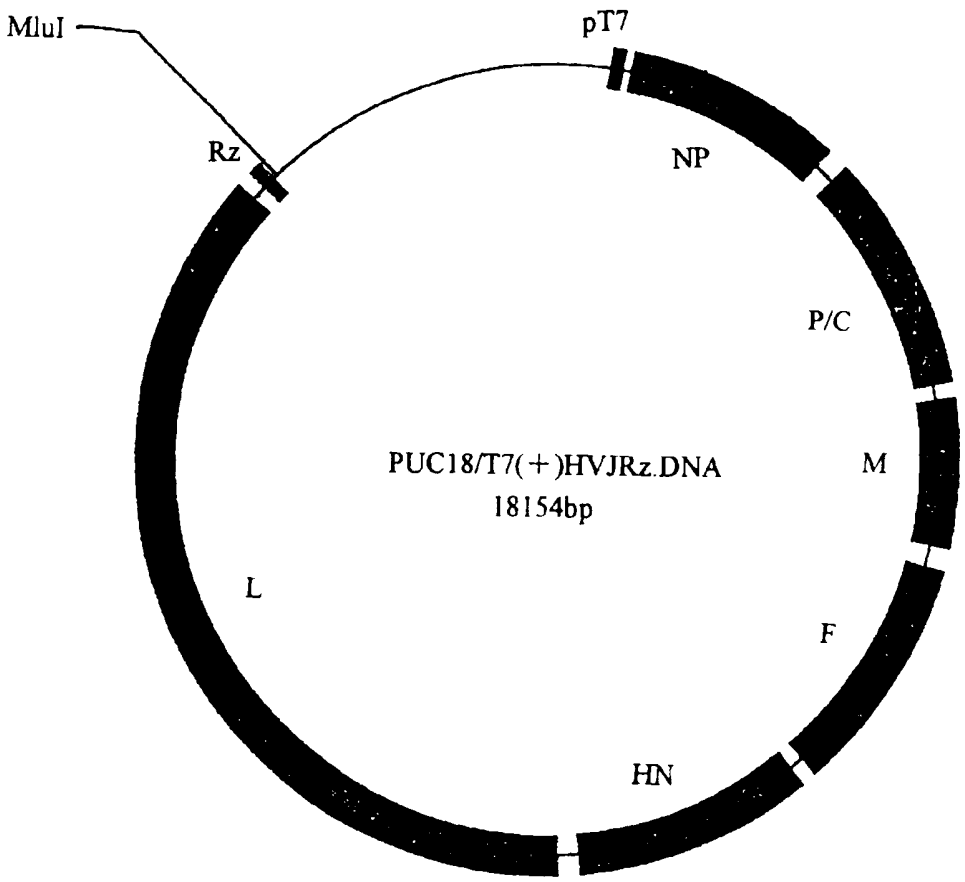
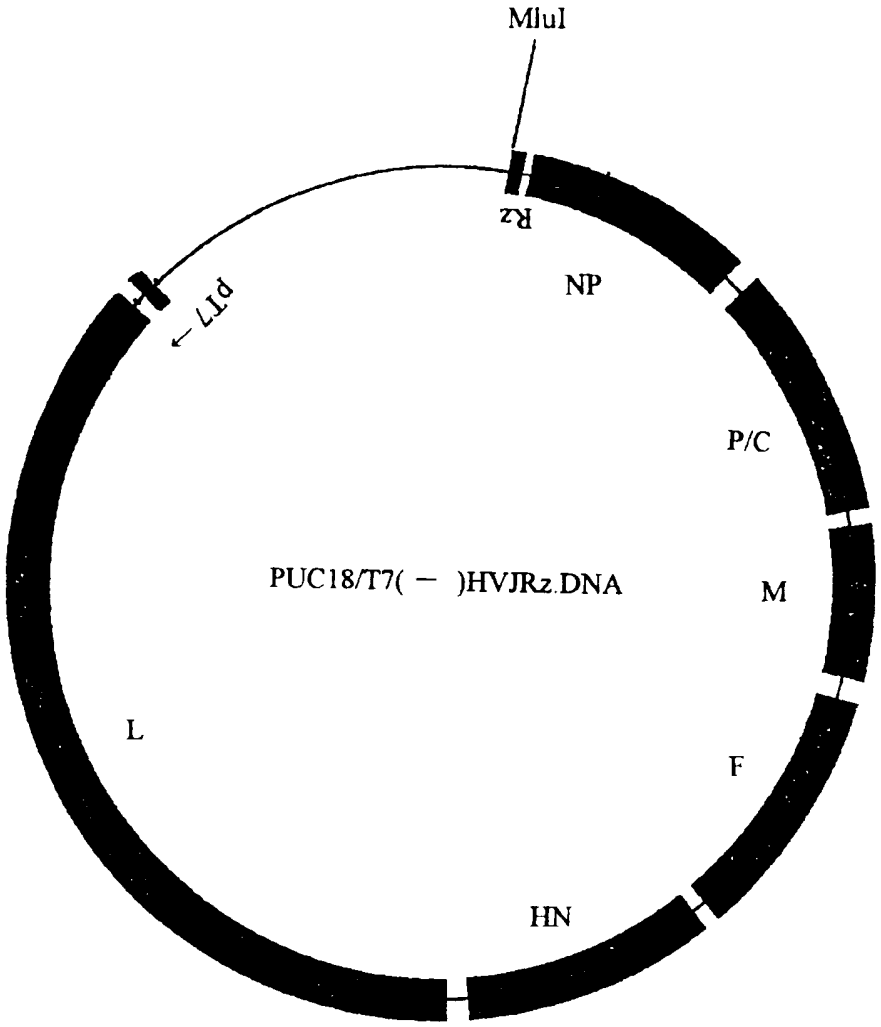


図 2



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(54) Title: VECTOR TO DELIVER AND EXPRESS FOREIGN GENE

(57) Abstract

A vector for delivering a foreign gene to a target cell for expression of the foreign gene is provided. The vector comprises a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus. The (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of the (-) sense RNA virus. Methods of preparing the vector are disclosed, as well as pharmaceutical compositions containing the vector, and methods of delivering the expression product of the foreign gene to a target cell.

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VECTOR TO DELIVER AND EXPRESS FOREIGN GENE

TECHNICAL FIELD

This invention relates to a delivery system which can be untilised to deliver and express a foreign gene in eukaryotic cells. In particular, the delivery system can be used to deliver an RNA gene which will direct synthesis of an encoded anti-sense RNA, catalytic RNA, peptide or polypeptide within specific target cells which can be of a selected type. The delivery system can be used *in vitro* to target eukaryotic cells in culture or can be used *in vivo* to deliver a prophylactic or therapeutic agent to specific cells in an animal or human that is diseased or infected or at risk of disease, infection or infestation.

BACKGROUND ART

Hitherto, conventional systems for delivery of therapeutic agents included pharmaceutical dosage forms such as capsules which are made principally of gelatin blends and which contain small amounts of other components such as dyes, plasticisers, preservatives and opaquing agents. These capsules function as a soluble external shell or envelope for delivery of drugs to a required location. Soft capsules are used for liquids while hard capsules are used for delivery of free flowing powders. Microencapsulation techniques are also well known. Other types of pharmaceutical dosage forms have included compressed tablets prepared by compaction of a formulation containing the drug and certain excipients selected to aid the processing and to improve the properties of the drug. These excipients can include binders, disintegrants, fillers, or diluents and lubricants. Film coated tablets are compressed tablets with a film coat applied. An example is an enteric coated tablet which allows the drug to be delivered to the intestines because the coating is insoluble in the stomach. Also known are sustained release tablets which allow release of the drug over a period of time.

In both dosage forms described above, the drugs normally reach the gastro-intestinal tract (GI tract) and diffuse across the gastro-intestinal membrane into the bloodstream. The drug contained in the tablet dosage form will disintegrate in the GI tract prior to entry into the bloodstream

due to the presence of the disintegrant and the capsule dosage form will dissolve prior to the drug entering the bloodstream.

However, a major problem in the delivery of drugs and other macromolecules into cells is the permeability barrier imposed by the plasma membrane. Pharmaceutical dosage forms comprising tablets or capsules are unable to penetrate the permeability barrier, especially in relation to macromolecules which can comprise polypeptides such as toxins, enzymes or antibodies, or polynucleotides such as DNA or RNA.

Various methods have been used for delivery of macromolecules into cells. These include physical treatments such as microinjection, permeabilisation by lytic agents or high voltage electric fields and induced uptake of calcium phosphate or polyethylene glycol co-precipitates. Cell entry by fusion of a delivery vehicle with the cell plasma membrane has been achieved by use of liposomes and reconstituted viral envelopes (RVEs). Live virus vectors and other engineered viral delivery vehicles have also been used. While many of these methods have been useful for *in vitro* delivery of macromolecules to cells in culture, few have been successfully applied to delivery of macromolecules *in vivo*.

Liposomes comprise artificial lipid envelopes which can be generated *in vitro* by condensation of phospholipid into a bilayer membrane which can enclose a soluble macromolecule. Trapping efficiency into liposomes can be as high as 20-30% but the efficiency of delivery of macromolecules is poor, especially *in vivo* where rapid clearance from the bloodstream and high uptake by the liver and spleen present difficulties. In general, liposomes do not allow specific cell targeting but covalent attachment of virus-specific antibodies to the liposome surface has been used to achieve delivery of macromolecules to virus infected cells *in vitro*.

RVEs comprise viral envelopes which have been formed by solubilising intact virus in detergent and reassembling the viral envelope on removal of the detergent. RVEs can be formed in the presence of therapeutic agents including macromolecules which become encapsulated and can be used for drug delivery *in vitro* and *in vivo*. Encapsulation efficiency for macromolecules is lower than for liposomes (3-5%) but delivery efficiency and

cell targeting are enhanced by the presence of viral spike glycoproteins in the RVE membrane. The spike glycoproteins recognise receptors in the plasma membrane of the target cell. Cells which lack the specific receptor are not recognised by the RVEs and so are not targeted for delivery. The spike glycoproteins also contain a fusion domain which enhances fusion of the RVE with the cell membrane. Methods for modifying the target specificity of RVEs by covalent attachment of various ligands to the spike glycoprotein have also been described and the use of genetically engineered chimeric attachment proteins containing specific surface receptor recognition domains has been suggested.

Live virus vectors have been used both *in vitro* and *in vivo* to deliver genes encoding prophylactic and therapeutic agents such as vaccine antigens and interleukins and to effect synthesis of the products in the target cells. A gene encoding the therapeutic agent is engineered into the viral genome and the product is expressed upon infection of target cells.

In live DNA virus vectors, the virus is engineered to contain a foreign gene or genes at a site in the genome which does not inhibit the infectivity of the virus. The virus can also be engineered to have reduced virulence for the target host. After infection of host cells, the virus expresses viral products as well as the foreign product. DNA viruses which have been engineered as live, replicating delivery vehicles include poxviruses herpesviruses, adenoviruses, papovaviruses, parvoviruses and baculoviruses of insects.

While live replicating virus vectors can be effective and efficient delivery vehicles, they are not usually acceptable for general human or veterinary use because of the risk of causing disease and because of potential environmental risks due to infection of non-target species. DNA viruses can also incorporate integration elements which can modify the host genetic structure and present the risk of inducing tumours and related disorders.

RNA viruses used for delivery of foreign genes to animal cells include retroviruses, alphaviruses, Semliki forest virus, Sindbis virus and influenza virus. Retrovirus vectors are usually constructed by transfection of helper cells with a

DNA molecule which contains the terminal domains (LTRs) and assembly elements (psi region) of a retrovirus and includes the coding region of a foreign gene. The helper cells express retrovirus structural proteins. The transfected DNA molecule is integrated into the DNA of the helper cells and an RNA molecule corresponding to a modified retrovirus genome is expressed. The modified genome including the foreign gene can be assembled into retrovirus-like particles by using the structural proteins expressed in the helper cells. Retrovirus vectors can be used for delivery of foreign genes into cells in the form of RNA which is transcribed into DNA and can be integrated into the host chromosomes and subsequently expressed by the host cell. Although retroviral vectors are a useful laboratory tool and have been used in particular cases for gene therapy, more general use is restricted by concerns that the host genetic structure can be modified resulting in tumours and related disorders.

The alphavirus Sindbis has been used as a delivery vehicle for expression of foreign genes in animal cells *in vitro*. Sindbis virus causes an acute febrile illness in humans and is transmitted by biting insects. Unlike retroviruses, the virus does not synthesise DNA or induce tumours in infected animals. Sindbis virus vectors have been constructed by deleting genes encoding the capsid structural proteins from the genome and substituting a foreign gene. However, as a (+) sense RNA virus, Sindbis does not carry a viral RNA transcriptase as a structural component of the particle. Efficient expression of the foreign gene in target cells requires expression of the viral replicase and transcriptase components which are encoded in the 5' two-thirds of the genome. Thus, the use of Sindbis virus expression vectors for delivery of therapeutic agents *in vivo* has three disadvantages: (i) the gene encoding the therapeutic agent cannot be delivered and expressed without prior expression of some viral proteins (replicase and transcriptase proteins); (ii) a limited amount of cloning capacity, approximately 3475 nucleotides, remains for insertion of foreign genes in the absence of infectious helper virus; and, (iii) the vector may become contaminated with wild type infectious virus due to recombination between the vector and

helper virus during vector preparation.

An influenza virus has been described in which the influenza A virus NS gene was replaced by a foreign indicator gene. When mammalian cells were transfected with the foreign gene, purified influenza virus polymerase complex and helper virus, recombinant virus particles were formed. As with the Sindbis virus vectors, the foregoing recombinant influenza virus vector has the disadvantages that the vector remains capable of expressing influenza proteins in the target cell and can revert to virulence by recombination with live virus.

Pattnaik and Wertz *(Proc. Natl. Acad. Sci. USA 88,* 1379-1383 (1991)) have described infectious defective interfering (DI) vesicular stomatitis virus particles produced by infecting cells with DI particles where the cells harboured vectors for the expression of all five vesicular stomatitis virus proteins. Such particles are not suitable for delivering a foreign gene to a target cell because of the infectivity of the particles.

Other recombinant (-) sense RNA virus particles have been described by Park *et al. (Proc. Natl. Acad. Sci. USA 88,* 5537-5541 (1991)) and Collins *et al. (Proc. Natl. Acad. Sci. USA 88,* 9663-9667 (1991)). These publications respectively describe Sendai virus particles and respiratory syncytial virus (RSV) particles which package a foreign gene. In both instances however, formation of the recombinant virus particles was dependent on co-transfection with live Sendai virus or RSV particles resulting in the production of infectious virus particles. The methods described by Park *et al.* and Collins *et al.* are thus not suitable for delivering a foreign gene to a target cell because of the risk posed by viral infection.

In vivo delivery of therapeutic proteins to keratinocytes using a retrovirus vector is known as is a drug delivery virion in a retrovirus envelope which contains a protein drug sequence useful as an anti-leukaemia and anti-tumour agent. Poxvirus expression systems for delivery of vaccine antigens and a system for delivery of genetic material into brain cells using a virus vector have also be described.

While the abovementioned prior art make it clear that viral vector delivery systems for foreign genes coding for a

protein therapeutic or prophylactic agent are not new, there remain difficulties associated with their general use *in vivo*. A live virus vector is not completely safe as it can revert to virulence, cause undesirable effects in the host or can be spread to non-target hosts. Non-replicating retroviral vectors present risks associated with alteration to the host chromosomes that can result in tumours and other available RNA virus delivery systems are limited in the scope of their application and have the disadvantage that they will express some viral products as well as the foreign gene.

The prior art includes descriptions of particles, referred to as virus-like particles (VLPs), which can be constructed by expressing viral structural genes in cultured eukaryotic cells. The procedure has been used to construct synthetic VLPs of several animal and human viruses. For example, the insertion of the complete polycistronic mRNA of poliovirus in the baculovirus polyhedrin gene has been reported. Insect cells infected with the recombinant baculovirus synthesised and processed the poliovirus polyprotein and generated "empty" poliovirus-like particles (VLPs). These synthetic "empty" capsids contained no RNA and were not infectious but were in some aspects similar to the complete virus. Similar methods have been used to construct core-like particles (CLPs) and VLPs of several other viruses including bluetongue, hepatitis B virus and bovine immunodeficiency virus. To date, the particles formed by this method have been generated by protein-protein interactions alone and have not contained defined molecules of nucleic acid (RNA or DNA). Hence, the technology has not yet been applied to the generation of VLPs of (-) sense RNA viruses which appear to require an RNA genome or genome fragment for initiation of the particle assembly process.

It has also been demonstrated that infectious viral particles can be recovered from cDNA clones representing the entire genome of some viruses. In this method cDNA is inserted into plasmid vectors containing promoters operative in eukaryotic cells. Transfection of eukaryotic cells with such vectors results in the production of infectious virus. This general approach has been used in relation to a number of viruses of humans, animals and plants including poliovirus,

Sindbis virus and brome mosaic virus. However, the method has only been applied to some DNA viruses and (+) sense RNA viruses with a genome that can function directly as an mRNA.

SUMMARY OF THE INVENTION

It is the object of this invention to provide an effective and completely non-infectious system for delivery of foreign genes to animal or human cells. The foreign gene will be in the form of a (-) sense RNA.

According to a first embodiment of this invention, there is provided a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus.

According to a second embodiment of this invention, there is provided a method of preparing a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, which method comprises the following steps:

i) preparing an expression vector incorporating a DNA molecule which contains DNA corresponding to said (-) sense RNA genome;

ii) introducing the expression vector prepared in step (i) into a eukaryotic host cell together with DNA for the expression of proteins for the formation of virus-like particles;

(iii) culturing the eukaryotic host cells under conditions which allow expression of said (-) sense RNA genome and said proteins, and incorporation of said (-) sense RNA genome into virus-like particles; and

(iv) harvesting said virus-like particles from the eukaryotic cell culture of step (iii).

According to a third embodiment of this invention,

there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient together with a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, and wherein said ribonucleoprotein complex includes a polymerase for synthesis of (+) sense RNA from said (-) sense RNA.

According to a fourth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to the first embodiment and co-transforming or co-transfecting said cell with a vector which provides an RNA-dependent RNA polymerase activity.

According to a fifth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to the first embodiment which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome.

According to a sixth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to cells of a tissue of a mammalian subject, said method comprising administering to said subject a vector according to the first embodiment which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome, or a pharmaceutical composition according to the third embodiment.

The (-) sense RNA genome of the vector of the first embodiment incorporates terminal fragments of the genome of a (-) sense RNA virus to facilitate packaging of the genome into the virus-like particles (VLPs). The VLPs contain the necessary viral proteins to target and enter specific cells and preferably

contains a protein to synthesize (+) sense RNA (ie. mRNA) transcripts of the foreign gene. The expression product of the foreign gene can be a peptide or polypeptide. The peptide expression product can be a biologically active molecule, specific therapeutic agent or immunogen. Similarly, the polypeptide expression product can be a biologically active protein, specific therapeutic agent or immunogen. The VLP vector also permits delivery of anti-sense RNA or catalytic RNA to a target cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the method of preparing the VLP vectors of the invention and the use of the VLP vectors for delivering a gene product to a target cell.

Figure 2 is a representation of a DNA construct comprising 5' and 3' domains, ribozyme domains R1 and R2 and a filler domain into which a foreign gene can be inserted at a preferably unique restriction endonuclease site such as the *NcoI* site shown.

Figure 3 depicts a process for preparing a genome construct comprising 5' and 3' domains, ribozyme domains and a filler domain.

Figure 4 depicts a process for preparing VLP particles from the genome construct resulting from the process depicted in Figure 3. The following abbreviations are used for restriction endonuclease sites: B, *BamHI*; E, *EcoRI*; P, *PstI*; and S, *SmaI*.

Figures 5a to 5d depict typical steps in the construction and cloning of a chimeric G protein gene. Figure 5a depicts the construction of an "anchor" gene fragment; Figure 5b the construction of a "donor" gene fragment; Figure 5c the construction of a chimeric G protein gene; and, Figure 5d depicts the cloning of the chimeric G protein gene.

Figure 6 is a schematic representation of the construction of baculovirus transfer vectors harbouring TB2-CAT genome constructs. The position of the polyhedron gene promoter and the direction of transcription therefrom in pAcYM1 and derivatives are indicated by the symbol "P" and the adjacent arrow respectively. The following abbreviations are used for restriction endonuclease sites: B, *BamHI*; N, *NcoI*; and V, *EcoRV*

Figure 7 presents nucleotide sequences of the CAT and

CAT3 PCR products with indication of the positions of the terminal and internal *NcoI* restriction enzyme sites. The CAT3 sequence is indicated in full with nucleotide differences in the CAT sequence shown above the sequence. The CAT sequence: (1) has a nucleotide modification at position 357 (T substituted for A) resulting in an amino acid change from Ile to Leu; and, (2) does not contain the rabies virus transcription termination/ polyadenylation sequence CATG$[A]_7$ immediately following the CAT3 gene translation stop codon (TAA).

BEST MODE AND OTHER MODES OF CARRYING OUT THE INVENTION

In the description of the invention set forth below, the following abbreviations are used:

CAT	chloramphenicol acetyl transferase
DIG	digoxigenin
EDTA	ethylenediaminetetraacetate
IPTG	isopropylthio-β-D-galactoside
LMT	low melting temperature
PCR	polymerase chain rection
TD	a solution of 0.8 mM tris-HCl (pH 7.4), 150 mM NaCl, 5 mM KCl and 0.7 mM Na_2 HPO_4 which is adjusted to pH 7.5 with HCl and autoclaved
VLP	virus-like particle
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

The term "foreign gene" is used in the following description and claims to denote a gene that is not normally present in the specific cells targeted by the VLP vector or if normally present in the specific cells, is not expressed at the level attainable after delivery of the foreign gene by the VLP vector of the invention.

In alternative nomenclatures used in relation to (-) sense RNA viruses, the M1, P and NS genes and respective expression products are equivalent as are the M2 and M genes and respective expression products.

A preferred process according to this invention, by which VLP vectors can be prepared and utilised to deliver the expression product of a foreign gene to a target cell, inlcudes the following steps:

(i) constructing a DNA molecule corresponding to a

modified genome or genome fragment of a (-) sense RNA virus where the DNA molecule contains a sequence corresponding to the coding region of a foreign gene or the coding regions of two or more foreign genes;

(ii) inserting the DNA molecule prepared in step (i) into an expression vector suitable for transfection of eukaryotic cells;

(iii) transfecting a eukaryotic cell with the recombinant expression vector prepared in step (ii) and simultaneously transfecting the same eukaryotic cell with vectors which express structural proteins of the (-) sense RNA virus and optionally with a vector for the expression of a protein with RNA-dependent RNA polymerase activity;

(iv) obtaining from the cell transfected in step (iii) virus-like particles (VLPs) consisting of a modified genome or genome fragment transcribed from the DNA molecule constructed in step (i), complexed with the viral proteins to form a ribonucleoprotein complex enclosed within a lipid envelope; and

(v) contacting a target cell with the VLPs produced in step (iv) to deliver the foreign gene expression product to the cells or preparing a composition for delivering the VLPs to target cells of tissue of an animal to deliver the foreign gene expression product to those cells.

Advantageously, the structural proteins and protein having RNA-dependent RNA polymerase activity referred to in step (iii) include those with similar functions to the L protein, G protein, N protein, M1 protein and M2 protein of rabies virus. The G protein can be a chimeric G protein incorporating a modified external domain. The VLP formed in step (iv) will thus consist of the modified genome or genome fragment transcribed from the DNA molecule constructed in step (i), complexed with the L protein and M1 protein and surrounded by a sheath of N protein in a ribonucleoprotein complex which is surrounded by an internal matrix comprising the M2 protein and enclosed within a lipid envelope including the G protein (or chimeric G protein incorporating a modified external domain).

The process is not limited to rabies virus however and the structural proteins, protein having RNA-dependent RNA polymerase activity and subgenomic (-) sense RNA fragments can

be obtained from any (-) sense RNA virus having either a segmented or non-segmented genome. Such viruses include, but are not limited to, viruses from the following families: Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Bunyaviridae, Arenaviridae and Filoviridae. The preferred viruses are viruses from the rhabdovirus and paramyxovirus genera.

A schematic representation of the process described in the preceding paragraphs is presented in Figure 1.

The DNA molecule referred to in step (i) above typically comprises domains containing DNA sequences corresponding to 5' terminal and 3' terminal non-coding regions of the particular (-) sense RNA viral genome in addition to the sequences corresponding to the coding regions of the one or more foreign genes. Preferably, the 5' and 3' domains are derived from the sequences of the 5' and 3' non-coding regions of the genome of a rhabdovirus or paramyxovirus.

Advantageously, the DNA molecule includes domains encoding ribozymes. The ribozyme domains can be constructed from any of the known ribozyme structures, some of which have been described by Haseloff and Gerlach (*Nature 334*, 585-591 (1988)). The ribozyme domains will be active during step (iii) of the above process and will ensure that the (-) RNA transcript expressed in eukaryotic cells will have a structure suitable for assembly of VLPs.

The foreign gene contained within the DNA construct can be the complete coding region of a selected foreign polypeptide including initiation and termination codons or can be a fragment of a gene corresponding to a functional domain or domains of a polypeptide. The polypeptide encoded by the foreign gene can be an immunogen, a therapeutically or biologically active peptide or polypeptide, or an engineered protein such as an antibody-like molecule. Alternatively, the foreign gene can encode anti-sense RNA or catalytic RNA directed against an intracellular RNA molecule. Multiple foreign genes can be inserted in tandem. A restriction enzyme site, *NcoI* for example, is advantageously included in a generic DNA construct to facilitate insertion of the selected foreign gene or genes to generate the DNA molecule.

The DNA molecule can also include a filler domain

comprising sequences of viral or other origin to give the construct sufficient length to be efficiently packaged in VLPs. The filler domain can constitute any nucleotide sequence that has characteristics which will allow the formation of VLPs. Preferably the filler domain will constitute a fragment derived from a portion of the L protein coding region of a rhabdovirus or paramyxovirus which is adjacent to the 5' terminal non-coding region of the (-) RNA genome. The filler domain will ensure that the genome to be expressed in step (iii) will be of sufficient size to allow formation of VLPs. That size is preferably greater than about 1000 nucleotides.

Advantageously, the DNA molecule incorporates cohesive ends suitable for insertion of the molecule at selected restriction enzyme sites of plasmid vectors.

In relation to step (i) of the process, a typical DNA construct is illustrated in Figure 2.

A DNA construct suitable for carrying a foreign gene is described in international application No. PCT/AU92/00363 (WIPO publication No. WO 93/01833), the entire disclosure of which is incorporated herein by cross-reference. That construct, TB-2, after incorporation into a eukaryotic expression vector as described in step (ii) above and used as described in steps (iii) to (v) above, allows the formation of rabies VLPs. Inclusion of a foreign gene or gene(s) at the *NcoI* site of the TB-2 construct permits construction by steps (i) to (v) above of a rabies VLP which can be used as a vector for delivery of the foreign gene into a eukaryotic cell for expression of the gene in that cell.

In TB-2, the 5' and 3' domains are derived from the known nucleotide sequence of the 5' and 3' terminal regions of the genome of rabies virus (PV and CVS strains). The R1 domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R1 ribozyme in the transcript will cleave the RNA to ensure that extraneous parts of the transcript are removed so that the 5' terminus of the transcript corresponds to, or approximates, that of the 5' terminus of the rabies virus genome. Similarly, the R2 ribozyme domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R2 ribozyme will cleave the RNA to

ensure that extraneous parts of the 3' region of the transcript (including the R2 domain) are removed so that the 3' terminus of the transcript approximates that of the 3' terminus of the rabies virus genome. The filler domain in the TB-2 construct is derived from the known nucleotide sequence of a 1167 nucleotide region at the 5' end of the rabies virus (PV strain) L protein gene. The TB-2 construct also includes an *NcoI* site at which any selected foreign gene or genes can be inserted. The preparation of a DNA molecule comprising a modified genome of a (-) sense RNA virus is illustrated in Figure 3 using, as an example, the TB-2 DNA molecule derived from the rabies virus genome. Use of such a molecule for the preparation of VLPs is illustrated in Figure 4.

According to the process illustrated in Figure 3, TB-2 DNA is constructed from 3 fragments (Fragment A, Fragment B, and Fragment C in Figure 3). Fragment A incorporates the 5' domain and R1 domain of TB-2 and can be prepared from overlapping complementary oligonucleotides. Suitable oligonucleotides are PJW.5R1A and PJW.5R1B, the sequences of which, together with other oligonucleotides suitable for use in other steps of the procedure, follow:

PJW.5R1A 5'-TACGTCACGCTTAACAAATAAACAACAAAAATGAGAAAAACAATCAAACA-ACTAGAGGTTCAGATTTAAG-3'

PJW.5R1B 5'-TACGTTTCGTCCTCACGGACTCATCAGACGCTTAATGAAAAAAACAAGAT-CTTAAATCTGAACCTCTAGT-3'

PJW.3R2A 5'-CATGGTAGGGGTGTTACATTTTTGCTTTGCAATTGACGCTGTCTTTTTCT-TCTCTGGTTTTGTTGTTAAGCGTC-3'

PJW.3R2B 5'-TTAAGCGTTTCGTCCTCACGGACTCATCAGACCGGCGAAAACACATCGCC-GGTGACGCTTAACAACAAAACCA-3'

PJW.L2R 5'-AGAGTGATAGATTTTGACTGA-3'

PJW.L4R 5'-AAATACATCACACAAGAGTCT-3'

Oligonucleotides PJW.5R1A and PJW.5R1B are annealed and end-filled using T4 DNA polymerase to produce a blunt-end double-stranded DNA molecule of the required nucleotide sequence which can then be cloned into, for example, the *SmaI* site of a suitable plasmid vector such as pBluescript IIKS+. The DNA can then be excised from the vector by using suitable restriction enzymes, *BamHI* and *EcoRI* for example, to generate the required fragment with cohesive ends in the required orientation

(Fragment A, Figure 3).

Fragment C incorporates the 3' domain, R2 domain and the foreign gene insertion site (*NcoI* site) of TB-2 and can be constructed from overlapping complementary oligonucleotide primers PJW.3R2A and PJW.3R2B. By a similar procedure to that described for the construction of fragment A, the oligonucleotides are annealed and end-filled using T4 DNA polymerase to produce a blunt-end double-stranded DNA molecule of the required nucleotide sequence which can be similarly cloned into, for example, the *SmaI* site of a vector such as pBluescript IISK+. The DNA can then be excised from the vector by using suitable restriction enzymes such as *BamHI* and *PstI*, to generate the required fragment with cohesive ends in the required orientation (Fragment C, Figure 3).

Fragment B incorporates the filler domain and can be constructed, for example, from the rabies virus (PV strain) genome using primer PJW.L2R (above) and reverse transcriptase to prepare a single-stranded cDNA copy of the required portion of the rabies L protein gene and then by using primers PJW.L2R and PJW.L4R (above) and the polymerase chain reaction (PCR) to amplify a double-stranded DNA molecule of the required nucleotide sequence. The DNA molecule can then be cloned into, for example, the *SmaI* site of a suitable plasmid vector such as pUC8. The DNA can then be excised from the vector using suitable restriction enzymes, *EcoRI* and *PstI* for example, to generate the required fragment with cohesive ends in the required orientation (Fragment B, Figure 3).

The TB-2 DNA construct can then be assembled by ligation of Fragment A, Fragment B and Fragment C with T4 DNA ligase to join the cohesive ends in the required orientation (Figure 3).

For the production of rabies VLPs, the TB-2 DNA construct is inserted into a vector for synthesis of (-) sense RNA. Advantageously, the (-) sense RNA is synthesised in an insect cell using a baculovirus expression vector. As shown in Figure 4, the TB-2 construct is inserted into a baculovirus transfer vector such as pAcUW31 at the *BamHI* site to form pAcUW31.TB2. Recombinant baculovirus capable of expressing TB-2 (-) sense RNA is formed by recombination in insect cells between

pAcUW31.TB2 and a baculovirus such as AcNPV to form AcNPV.TB2. However, the transfer vector may be any transfer vector containing baculovirus promotors, such as Pol and p10.

Production of the rabies virus VLPs containing a (-) sense RNA modified genome or genome fragment are produced by co-infection of an insect cell with the recombinant baculovirus AcNPV.TB2 and other recombinant baculoviruses which express rabies virus L protein, G protein, N protein, M1 protein and M2 protein as shown in Figure 4.

The procedure for preparation of recombinant baculoviruses which express rabies virus G and N proteins has been described in Prehaud *et al.* (1989) *Virology 173*, 390-399 and Prehaud *et al.* (1990) *Virology 178*, 486-497, and the baculovirus expression of rabies N, M1, M2 and G proteins is disclosed in Prehaud *et al.* (1992) *Virology 189*, 766-770, the entire disclosures of which are incorporated herein by cross-reference. Similar procedures can be used to prepare recombinant baculoviruses which express the rabies virus L protein in insect cells. The sequence of the L gene has been disclosed in Tordo *et al.* (1988) *Virology 165*, 565-576. From known gene sequences, a person of skill in the art can readily prepare vectors for the expression of proteins from other (-) sense RNA viruses.

As indicated above in step (i) of the overall process of the invention, one or more foreign genes are included in the DNA molecule. Using the TB-2 construct as an example, the construct can be modified to incorporate any selected foreign gene or genes by insertion of the selected gene or genes at the *NcoI* site. By using the *NcoI* site, the selected gene or genes can be positioned within the construct so that the initiation codon will substitute for the initiation codon of the nucleoprotein (N) gene of the virus from which the terminal domains are derived. However, the foreign gene or genes can be inserted at any suitable site within the filler domain, if present, or proximal the DNA sequences corresponding to the 5' or 3' domains of the (-) sense RNA genome.

Preferably, DNA comprising the foreign gene includes the initiation codon, termination codon and coding region of the selected foreign gene, all or a part of the 3' noncoding region

including the polyadenylation site of the rabies virus N protein mRNA, or equivalent sequence, and cohesive ends suitable for insertion of the DNA into the DNA molecule. As an example of the last mentioned feature, the DNA will have *NcoI* restriction termini for insertion of the DNA at the *NcoI* site of the TB-2 construct. It will be understood by one of skill in the art that the DNA comprising the foreign gene is inserted into the DNA molecule so that (+) sense RNA formed in a target cell contains the sense strand of the foreign gene.

The foreign gene can be obtained by established procedures of molecular cloning well known in the art. Addition of the 3' noncoding region, polyadenylation site and cohesive ends can be conducted, for example, by using PCR and suitable oligonucleotide primers which contain the desired sequences. Other methods of modification that are known in the art can also be used, such as ligation of oligonucleotide linkers to DNA comprising the gene.

It will be appreciated that the process described above for the production of rabies VLPs can be applied to any other (-) sense unsegmented RNA virus, particularly rhabdoviruses and paramyxoviruses. Essentially, the required VLP will contain a suitably modified genome or genome fragment containing a foreign gene or genes including essential assembly and transcription signals provided by the 5' and 3' domains of the DNA construct. Ribozyme domains R1 and R2 can be provided to ensure that the RNA transcript has suitable terminal sequences. The selected foreign gene can be inserted at any suitable site within the DNA construct. The RNA transcript of the DNA construct when co-expressed in eukaryotic cells with the structural proteins of the homologous (-) sense RNA virus is incorporated in a VLP.

As described in step (v) of the process of this invention, a VLP vector comprising a (-) sense genome which includes a foreign gene can be used to deliver the foreign gene to a eukaryotic cell and to express the polypeptide product or RNA of the foreign gene in the target cell.

It will also be appreciated that delivery and expression of the foreign gene in a eukaryotic cell will occur by adsorption of VLPs to specific receptors on the cell surface

and subsequent entry of the VLPs into the cytoplasm of the cell. Advantageously, expression of the foreign gene occurs by virtue of components of the ribonucleoprotein complex of the VLP which are activated in the cytoplasm of the target cell. In particular, the ribonucleoprotein complex contains an RNA-dependent RNA-polymerase such as the L protein of rabies virus. The presence of an RNA-dependent RNA-polymerase in the ribonucleoprotein complex is not essential however and the activity can be provided by co-transfection of the target cell with a vector from which an RNA-dependent RNA-polymerase activity is expressed. The vector may be a plasmid or a virus. Typically, the vector is an homologous (-) sense RNA virus.

It is known that recognition of and entry into cells by viruses is a function of the envelope glycoproteins on the viral surface. In the case of rabies virus and other (-) sense RNA viruses, this function is served by the G protein. The target cell specificity of the VLP vectors of the present invention can therefore be changed by modifying the structure of the envelope glycoprotein. This can be achieved by constructing chimeric envelope protein genes which can be substituted for the envelope glycoprotein gene(s) during step (iii) of the process described above. Methods for the construction and expression of chimeric viral glycoproteins are known and are described, for example, by Puddington *et al.* (*Proc. Natl. Acad. Sci USA 84*, 2756-2760 (1987)), Schubert *et al.* (*J. Virol. 66*, 1579-1589 (1992)) and Owens and Rose (*J. Virol. 67*, 360-365 (1993)).

A suitable method for the construction of a chimeric glycoprotein gene is illustrated in Figures 5a to 5d. In this illustration, the nucleotide sequence of a chimeric glycoprotein is constructed, for example, from the envelope glycoprotein genes of rabies virus and the rhabdovirus, vesicular stomatitis virus (VSV). The chimeric gene illustrated retains internal and transmembrane domains of the rabies glycoprotein but includes the external domain of VSV. The chimeric gene components are advantageously synthesised by PCR amplification of template DNA using oligonucleotide primers. Such primers are shown as "OLIGO 21" to "OLIGO 24" in Figures 5a and 5b. The chimeric glycoprotein gene can be substituted for the rabies G protein gene in an expression vector, a recombinant baculovirus for

example, and used for the construction of rabies VLP vectors. VLPs formed using such a chimeric structure will adsorb to and enter cells recognised by the VSV glycoprotein. Such a process can be used to construct chimeric envelope proteins which incorporate any selected external domain which can be included in the surface structure of the VLPs. The chimeric structure can be selected so that the VLPs can adsorb to, enter and express the foreign gene in specific cells which carry a receptor for the modified external domain.

External domains that can be used to alter the target cell specificity of the VLP vectors of this invention include, but are not limited to, the external domains of influenza virus hemagglutinin, human immunodeficiency virus (HIV) gp160, and paramyxovirus hemagglutinin-neuraminidase. Alternatively, the chimeric envelope protein can comprise an external domain from a virus fused to the trans-membrane and internal domains of the virus on which the VLP is based, wherein the first mentioned virus is different to the second mentioned virus.

In relation to step (ii) of the process described above, it will be appreciated by persons skilled in the art that any suitable vector-host cell system can be used to express the modified genome or genome fragment and viral proteins for VLP formation. Suitable host cells include higher eukaroytic cells such as vertebrate cells using poxvirus, papillomavirus or retrovirus vectors, or lower eukaryotic cells such as yeast cells. The preferred expression system is, however, an insect host cell such as *Spodoptera frugiperda* harbouring a recombinant baculovirus vector.

Following similar methods to those described above for expressing rabies VLPs based on the TB-2 construct, other (-) sense RNA genes can be expressed in insect cells using baculovirus vectors. Simultaneous expression of a genome construct as a (-) sense RNA transcript and homologous (-) sense RNA virus structural proteins in insect cells allows formation of VLPs.

For delivering a foreign gene to target cells of a subject, the VLP vectors of the invention may be administered as follows: by topical treatment of mucous membranes; by intramuscular, subcutaneous, intraperitoneal or intravenous

injection into tissue; or, by delivery to the intestinal mucosa either naked or in acid- and pepsin-resistant capsules. Examples of topical treatment of mucous membranes are oral, nasal, occular, respiratory, anal, vaginal or urethral routes. Alternatively, the VLP vectors may be administered to cells or tissue *in vitro* by directly contacting the cells or tissue with the VLPs.

Pharmaceutical preparations of the VLP vectors of the invention are prepared by combining the VLPs with pharmaceutically acceptable carriers, diluents, adjuvants or excipients or combinations thereof.

The number of VLPs administered to a target cell or target cell of a tissue will depend on the expression product of the foreign gene. In some instances a single VLP per cell will be sufficient whereas in other instances a large number of VLPs will be required per cell, such as, where the foreign gene expression product is an anti-sense RNA. One of skill in the art would be able to determine the number of VLPs to be administered from a consideration of the expression product of the foreign gene.

By using the process described in this invention, and illustrated using the rhabdovirus TB-2 genome, synthetic (-) sense RNA virus VLPs can be produced without helper virus, defective-interfering particles or synthetic transcription complexes. The VLPs synthesised by this process are modified to contain a foreign gene which can be delivered to and expressed in eukaryotic cells. The VLP vectors of the invention can be modified to include an external domain whih allows adsorption to and entry into cells of a selected type. The VLPs do not contain complete genes from the homologous (-) sense RNA virus so the synthetic particles are non-infectious.

The invention will now be illustrated by the following non-limiting examples. Except as otherwise noted, standard methods were used for the isolation and manipulation of nucleic acids described, for example, by Sambrook *et al.* in *Molecular Cloning: a Laboratory Manual* 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (1989).

EXAMPLE 1

Preparation of recombinant plasmids containing VLP genome

constructs incorporating the chloroamphenicol acetyl transferase (CAT) reporter gene.

In this example, construction of plasmids comprising a sub-genomic fragment of rabies virus harbouring a foreign gene is described. The TB-2 construct described in international application No. PCT/AU92/00363 was utilised as a sub-genomic fragment of rabies virus and the CAT reporter gene employed as a foreign gene.

Plasmids used for constructing the recombinant plasmids were obtained from the following sources: pSVL-CAT (Cameron and Jennings (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9139) was obtained from CSIRO Division of Biomolecular Engineering, North Ryde, NSW, Australia; pBluescript KSII+ was obtained from Promega Corporation, Madison, WI, USA; and, pAcYM1 (Matsuura *et al.* (1987) *J. Gen. Virol.* 68, 1233) was obtained from the Institute of Virology and Environmental Microbiology, Oxford, UK.

Plasmid pTB2 is described in international application No. PCT/AU92/00363. The BamHI TB-2 insert of pTB2 is also contained within the pAcTB2 vector described in PCT/AU92/00363. pAcTB2 has been deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, NSW 2073, Australia, under accession No. 92/32588.

Oligonucleotide primers were synthesised for PCR amplification of required DNA fragments (CPR1, CPR2 and CPR3) and DNA sequencing (Bac1 and Bac2). CPR1, CPR2 and CPR3 contained the terminal sequences of the CAT gene, *Nco*I restriction endonuclease sites to facilitate subcloning into the *Nco*I site of pTB2 and in the case of CPR3, the rabies virus N-gene transcription termination/ polyadenylation sequence ($CATG[A]_7$). The sequence of each oligonucleotide primer follows:

CPR1 5'-CCCCATGGAGAAAAAAATCACTGGAT-3'

CPR2 5'-GGCCATGGTTACGCCCCGCCCTGC-3'

CPR3 5'-GGCCATGGTTTTTTTCATGTTACGCCCCGCCCTGC-3'

Bac1 5'-TTACTGTTTTCGTAACA-3'

Bac2 5'-CGCACAGAATCTAGCGC-3'.

A. Construction of plasmids pAcTB2-CAT and pAcTB2-CAT-R.

A full-length copy of the CAT gene was obtained by PCR using primers CPR1 and CPR2 and plasmid pSVL-CAT DNA as a

template for amplification. The reaction was performed using *Taq* Buffer, 3.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5 units of *Taq* DNA polymerase (Promega Corp.), 1 µg of each primer and 7.5 ng of pSVL-CAT plasmid DNA. The reaction mixes were heated at 85°C, 3 min before the addition of *Taq* DNA polymerase and subjected to 40 cycles at 95°C for 90 s, 51°C for 90 s, 72°C for 90 s followed by incubation at 72°C for 5 min before maintainance at 25°C until DNA products were processed.

The CAT DNA product was applied to a 0.8% LMT agarose gel and a discrete DNA band of approximately 0.7 kb was excised. An equal volume of TE buffer pH 7.6 was added and the mixture was incubated at 68°C for 5-8 min with occasional vortexing. The DNA was extracted once with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and was precipitated by the addition of 0.3 M sodium acetate pH 5.2, 20 mg glycogen (Boehringer Mannheim) as a carrier and 2.5 vol of ethanol. After incubation at -20°C for 30 min, DNA was collected by microcentrifugation, washed with 70% ethanol and dried under vacuum.

3'-Terminal adenosine overhangs resulting from *Taq* DNA polymerase extension were removed using the 3'->5' exonuclease activity associated with the Klenow fragment of DNA polymerase 1. Purified DNA products were reacted with 2.5 units of Klenow fragment (Promega Corp.) in restriction enzyme buffer H (Boehringer Mannheim) at 22°C for 15 min and extracted with phenol:chloroform:isoamyl alcohol and precipitated as above.

The CAT gene DNA was blunt-end ligated into the dephosphorylated *Eco*RV site of pBluescript KSII+ (Stratagene) followed by transformation of XL1-Blue *E. coli* host cells (Stratagene) and the selection of ampicillin-resistant white colonies on agar plates prepared with TYM medium and containing ampicillin, X-gal and IPTG. Plasmids containing the CAT gene were identified and the sequences of the inserts in pBlue-CAT were determined using T3 and T7 sequencing primers (Promega Corp.) and Sequenase™ (United States Biochemicals) sequencing reagents.

Plasmid pBlue-CAT was subjected to partial digestion with *Nco*I and the resulting DNA fragments were resolved in a 1.2% LMT agarose gel. The full-length CAT gene fragment of

approximately 0.7 kb was isolated, purified and subcloned into the dephosphorylated *NcoI* site of pTB2 as described above with the exception that no blue/white selection system was available. Plasmids containing inserts were identified and sequenced using the T7 sequencing primer to determine the orientation of inserts in the *NcoI* site of the TB2 genome construct. Two clones containing the CAT gene in forward (pTB2-CAT) and reverse (pTB2-CAT-R) orientation were selected as shown in Figure 6.

Plasmids pTB2-CAT and pTB2-CAT-R were digested with *BamHI* to obtain inserts containing the TB2 DNA construct incorporaiting the CAT gene in both orientations. The inserts of approximately 2.1 kb were isolated and purified as above and subcloned into the dephosphorylated *BamHI* site of the baculovirus transfer vector pAcYM1. Recombinant plasmids were identified and the orientation of the inserts was determined by sequencing as described above with primers Bac1 and Bac2 which allowed strand extension across the two reformed *BamHI* sites of the recombinant pAcYM1 vector. The sequences of the Bac1 and Bac2 primers are shown above. Clones possessing inserts in the required orientation, pAcTB2-CAT and pAcTB2-CAT-R, were selected and plasmid DNA purified by CsCl gradient centrifugation.

The construction of pAcTB2-CAT and pAcTB2-CAT-R is shown schematically in Figure 6.

B. Construction of plasmids pAcTB2-CAT3 and pAcTB2-CAT3-R.

Plasmids pAcTB2-CAT3 and pAcTB2-CAT3-R were prepared exactly as described above for plasmids pAcTB2-CAT and pAcTB2-CAT-R except that the CAT gene was obtained from plasmid pSVL-CAT DNA by PCR amplification using primers CPR1 and CPR3. This resulted in the inclusion of the rabies virus polyadenylation sequence ($CATG[A]_7$) immediately after the CAT gene termination codon in addition to the polyadenylation sequence present in TB-2.

The sequence of the CAT gene inserts in plasmids pTB2-CAT and pTB2-CAT3 are shown in Figure 7. Plasmid and primer sequences outside of the terminal *NcoI* restriction endonuclease sites are not presented.

EXAMPLE 2

Construction of recombinant baculoviruses containing TB2-CAT genome constructs.

The baculovirus AcPAK6 was grown in *Spodoptera frugiperda* (Sf9) cells and purified by sucrose gradient centrifugation. DNA was isolated and purified by CsCl gradient centrifigation and digested to completion with *Bsu*36I. Sf9 cells were co-transfected with 100 ng *Bsu*36I-linearized AcPAK6 DNA and 1 µg of each of the four plasmid constructs (pAcTB2-CAT, pAcTB2-CAT-R, pAcTB2-CAT3 and pAcTB2-CAT3-R) using Lipofectin™ (Gibco/BRL) transfection reagent. Cells were incubated at 28°C for 4 days and recombinant baculoviruses were identified by plaque selection. Cells were treated with X-gal to differentiate wild type (blue) from recombinant (white) baculovirus clones and the plaques were visualized by staining with neutral red. Clearly defined white plaques were selected and grown in duplicate 96-well cultures of Sf9 cells at 28°C for 3-6 days.

Cell lysates were prepared from one set of duplicate cultures after 3 days for hybridization analyses to confirm integration of the four TB2-CAT genome constructs. A digoxigenin (DIG)-labelled probe was prepared by PCR using 12 ng gel-purified CAT3 PCR product as a template, 0.5 µg CPR1 and CPR3 primers, reaction mixes containing 4mM $MgCl_2$, 0.5 mM dATP, dCTP and dGTP, 0.32 mM dTTP, 8 nmol DIG-11-dUTP (Boehringer Mannheim), 2.5 units *Taq* DNA polymerase (Promega Corp.) and the cycling temperatures described above. Dot hybridizations with the DIG-labelled CAT probe identified recombinant baculoviruses containing the TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R genome constructs.

After 4 days, a portion of the medium from duplicate cultures infected with recombinant baculoviruses identified as containing the required genome constructs was used to infect 24-well cultures of Sf9 cells to produce virus seed stocks for subsequent infection of larger cell cultures. Cultures of Sf9 cells were infected as a source of DNA for PCR amplification to confirm that complete rather than truncated DNA constructs had integrated into the recombinant baculoviruses. DNA obtained from cell lysates was used as a template for PCR amplification with the Bac1 and Bac2 primers described above. Amplification products were resolved in 0.8% agarose gels and fragments of the appropriate size, approximately 2.1 kb, were identified

confirming that the recombinant baculoviruses contained full length TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R constructs.

To produce cloned virus stocks, virus seed stocks from the 24-well cultures were subjected to a second round of plaque purification in Sf9 cells. Well- separated plaques were selected, isolated and used to produce stocks of cloned recombinant baculoviruses for use in subsequent manipulations.

EXAMPLE 3

Preparation of rabies VLPs containing TB2-CAT genome constructs.

Recombinant baculoviruses expressing rabies virus structural proteins (N/M1 and M2/G in dual expression vectors) and each of the four recombinant baculoviruses expressing genome constructs TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R were used to infect spinner cultures of Sf9 cells. Cultures were incubated at 28°C for 3 days, the medium harvested, clarified by centrifugation and VLPs were collected by ultracentrifugation at 27000 rpm for 1 h at 4°C in a Beckman SW28 rotor. VLPs were resuspended in TD buffer supplemented with 1 mM EDTA and centrifuged through TD-buffered 10% (w/w) sucrose onto a cushion of TD-buffered 40% (w/w) sucrose at 35000 rpm for 30 min at 4°C in a Beckman SW40Ti rotor. The band at the interface was harvested, diluted and the semi-purified VLPs collected by centrifugation at 30000 rpm for 90 min at 4°C in a Beckman SW40Ti rotor.

VLP formation was demonstrated by SDS-PAGE of disrupted pellets and Western blotting using polyclonal rabies virus antiserum. Rabies virus structural proteins G, N, M1 and M2 were identified in VLPs produced with all four TB2 genome constructs containing the CAT gene - TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R. Visual comparison of the intensity of the structural proteins in VLPs produced using the four TB2-CAT genome constructs with that observed for VLPs produced with the TB2 genome suggested that the Sf9 cells shed similar quantities of VLPs irrespective of the nature of the TB2 genome employed.

EXAMPLE 4

Detection of CAT gene expression.

In this example, expression of the CAT reporter gene in target cells transfected with the VLPs prepared in Example 3

is described. As the VLPs did not contain the L gene product, cells were co-transfected with live rabies virus to provide RNA-dependent RNA-polymerase activity. The (-) sense RNA of the VLPs was thus converted to (+) sense RNA allowing CAT expression.

Experiment 1.

Monolayers of 5 x 10^5 baby hamster kidney cells (BHK-21, BSR clone) were infected with 8 x 10^6 plaque-forming units of rabies virus (CVS strain). At 4 hours post-infection, the infected monolayers and uninfected BHK-21 cell monolayers were treated with 2 x 10^9 VLPs containing the following genome constructs: (a) TB2-CAT; (b) TB2-CAT3; (c) TB2-CAT-R; (d) TB2-CAT3-R; or, (e) no VLPs. At 2 days post-infection all monolayers were harvested and assayed for CAT gene expression by using the CAT-ELISA (Boehringer Mannheim).

Experiment 2.

In a second experiment, BHK-21 cell monolayers were treated as described in Experiment 1 except that rabies virus infections and VLP treatments were conducted simultaneously: that is, a 4 hour interval was not allowed between infection and VLP treatment.

The results of both experiments are presented in Table I.

TABLE I.

CAT gene expression from VLPs in BHK-21 cells (BSR clone) as detected by CAT-ELISA.

VLP Genome	Quantity of CAT expressed (pg)			
	Experiment 1		Experiment 2	
	Rabies-infected cells	Uninfected cells	Rabies-infected cells	Uninfected cells
TB2-CAT	15	0	15	0
TB2-CAT3	15	0	15	0
TB2-CAT-R	0	0	0	0
TB2-CAT3-R	0	0	0	0
No VLPs	0	0	0	0

The results presented in Table I demonstrate that a foreign gene, in this case CAT, can be expressed from sequence information contained within a (-) sense VLP genome. Expression was dependent on correct orientation of the foreign gene with CAT being detectable only in those cells transfected with TB2-CAT and TB2-CAT3 VLP genomes. Expression was also dependent on viral RNA-dependent RNA-polymerase activity as CAT was only detectable in cells infected with rabies virus (left-hand column for each experiment in Table I).

It will be appreciated that administration of the VLPs of the invention which can express an immunogenic protein to animals or humans who are at risk of disease, infection or infestation will cause immunity in much the same way as existing vaccines incorporating inactivated or attenuated viruses. However, there will be advantages because there is no possibility that infectious virus will be present or that reversion to virulence will occur because the VLPs of the present invention use only a fragment of the viral genome. Similarly, the VLPs of this invention can be used to deliver therapeutic agents to diseased or infected tissue. However, unlike other delivery systems presently available, the VLPs can be targeted to specific cells or tissues, can allow synthesis and hence amplification of the therapeutic agent in the target tissue, are completely non-infectious and typically do not carry genes of an infectious agent. Moreover, as the VLPs contain no DNA, no integration elements and no enzyme capable of DNA synthesis, there is no risk of modification of the host genome which can result in the induction of tumours or related disorders.

It will also be appreciated that many modifications can be made to the invention described above without departing from the broad scope and ambit thereof.

DEPOSITION OF MATERIAL ASSOCIATED WITH THE INVENTION

A sample of plasmid pAcTB2 was deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, NSW 2073, Australia, on 15 September 1992 and given the accession number 92/32588.

CLAIMS

1. A vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus.

2. A vector according to claim 1 which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome.

3. The vector according to claim 2 wherein said (-) sense RNA virus is a rhabdovirus or paramyxovirus.

4. The vector according to claim 3 wherein said (-) sense RNA virus is rabies virus.

5. The vector according to claim 4 wherein:

said (-) sense RNA genome comprises a 5' domain from the genome of rabies virus, a filler domain comprising rabies virus genomic RNA, said one or more foreign genes and a 3' domain from the genome of rabies virus;

said ribonucleoprotein complex comprises said (-) sense RNA genome together with rabies M1 and L proteins surrounded by a sheath of rabies N protein; and

said ribonucleoprotein complex is surrounded by an internal matrix comprising rabies M2 protein and is enclosed in a lipid envelope including rabies G protein.

6. A vector according to claim 1 wherein said virus-like particle includes modified glycoprotein comprising an external domain which targets said virus-like particle to a selected cell type.

7. The vector according to claim 6 wherein said modified glycoprotein comprises the internal and transmembrane domains of rabies virus G protein fused to an external domain comprising a polypeptide ligand for a receptor on the surface of said selected cell type.

8. The vector according to claim 1 wherein the expression product of said foreign gene is selected from the group consisting of a peptide, a polypeptide, an anti-sense RNA and a catalytic RNA.

9. A method of preparing a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, which method comprises the following steps:

i) preparing an expression vector incorporating a DNA molecule which contains DNA corresponding to said (-) sense RNA genome;

ii) introducing the expression vector prepared in step (i) into a eukaryotic host cell together with DNA for the expression of proteins for the formation of virus-like particles;

(iii) culturing the eukaryotic host cells under conditions which allow expression of said (-) sense RNA genome and said proteins, and incorporation of said (-) sense RNA genome into virus-like particles; and

(iv) harvesting said virus-like particles from the eukaryotic cell culture of step (iii).

10. The method according to claim 9 wherein said DNA molecule includes at least one ribozyme domain which cleaves the initial (-) sense RNA transcript formed in step (iii) to provide a molecule which can be incorporated into said virus-like particles.

11. A method according to claim 10 wherein said DNA molecule includes two ribozyme domains which cleave the initial (-) sense RNA transcript to, provide a molecule having 5' and 3' ends which approximate the 5' and 3' ends of the genome of said (-) sense RNA virus.

12. The method according to claim 9 wherein said expression vector is derived from a baculovirus.

13. The method according to claim 12 wherein said baculovirus is AcNPV or AcPAK6.

14. The method according to claim 9 wherein said eukaryotic host cell is an insect cell.

15. The method according to claim 14 wherein said insect cell is *Spodoptera frugiperda*.

16. A pharmaceutical composition comprising a

pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient together with a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, and wherein said ribonucleoprotein complex includes a polymerase for synthesis of (+) sense RNA from said (-) sense RNA.

17. A method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to claim 1 and co-transforming or co-transfecting said cell with a vector which provides a RNA-dependent RNA polymerase activity.

18. A method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to claim 2.

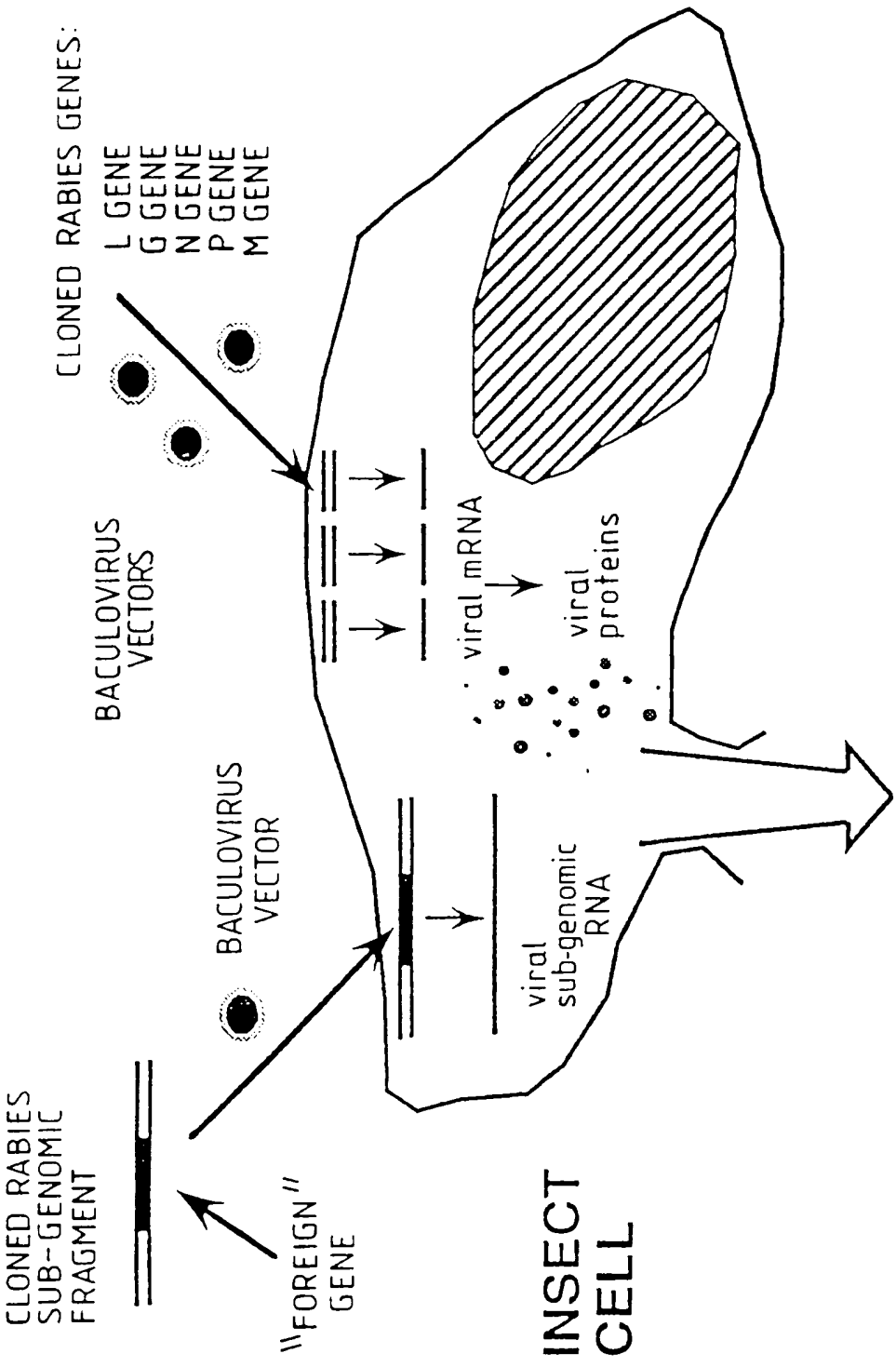
19. A method of delivering the expression product of a foreign gene to cells of a tissue of a mammalian subject, said method comprising administering to said subject a vector according to claim 2 or a pharmaceutical composition according to claim 16.

20. The method according to claim 19 wherein said delivery of the expression product of a foreign gene is for the treatment of a disease state or a pathological condition.

21. The method according to claim 19 wherein said virus-like particle includes modified glycoprotein comprising an external domain which targets said virus-like particle to a selected cell type.

22. The method according to claim 21 wherein said modified glycoprotein comprises the internal and transmembrane domains of rabies virus G protein fused to an external domain comprising a polypeptide ligand for a receptor on the surface of said selected cell type.

Fig.1.


CLONED RABIES GENES:
L GENE
G GENE
N GENE
P GENE
M GENE
BACULOVIRUS VECTORS
BACULOVIRUS VECTOR
CLONED RABIES SUB-GENOMIC FRAGMENT
"FOREIGN" GENE
viral mRNA
viral proteins
viral sub-genomic RNA
INSECT CELL

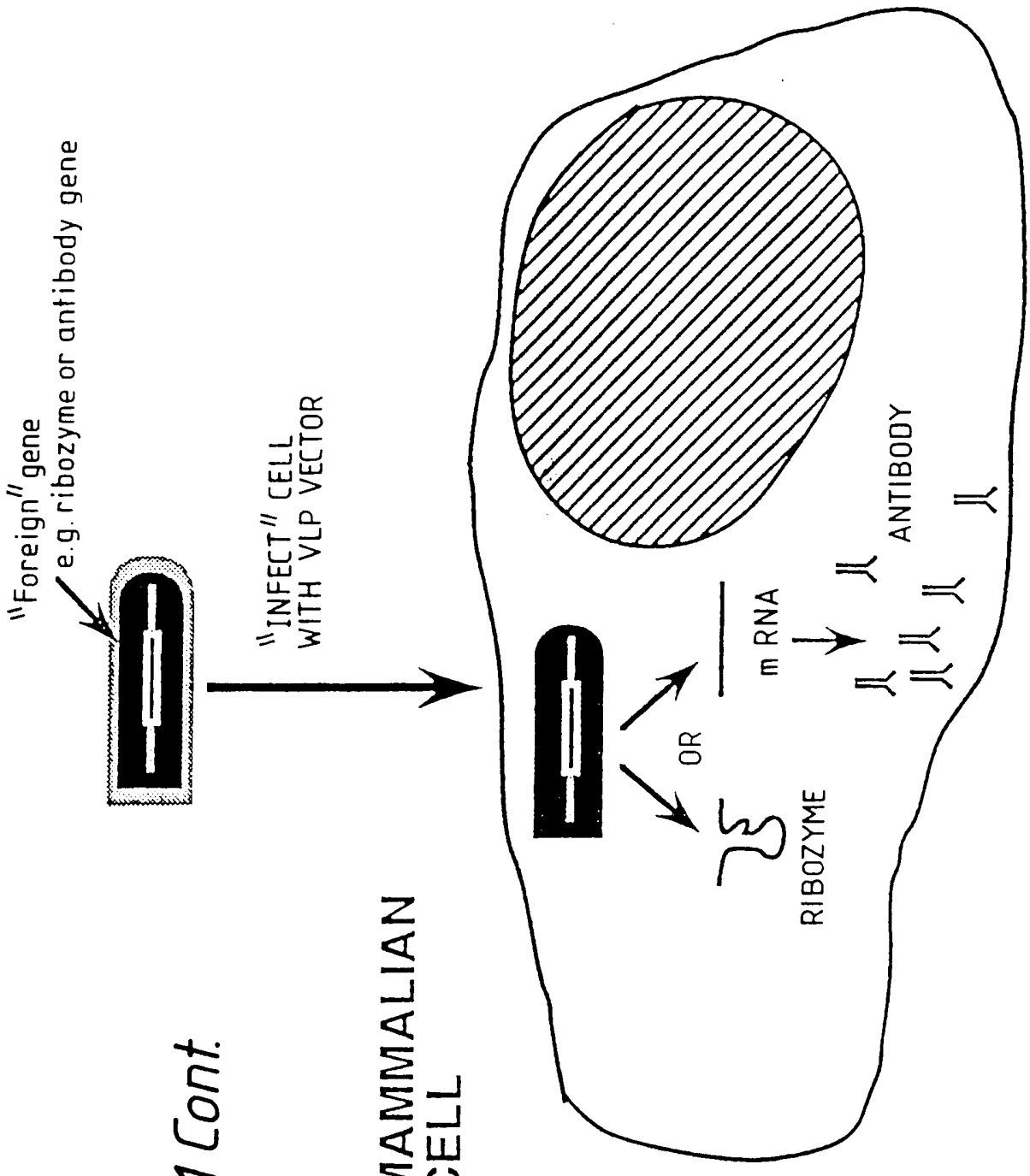

"Foreign" gene
e.g. ribozyme or antibody gene
"INFECT" CELL
WITH VLP VECTOR
MAMMALIAN
CELL
OR
mRNA
RIBOZYME
ANTIBODY

Fig. 1 Cont.

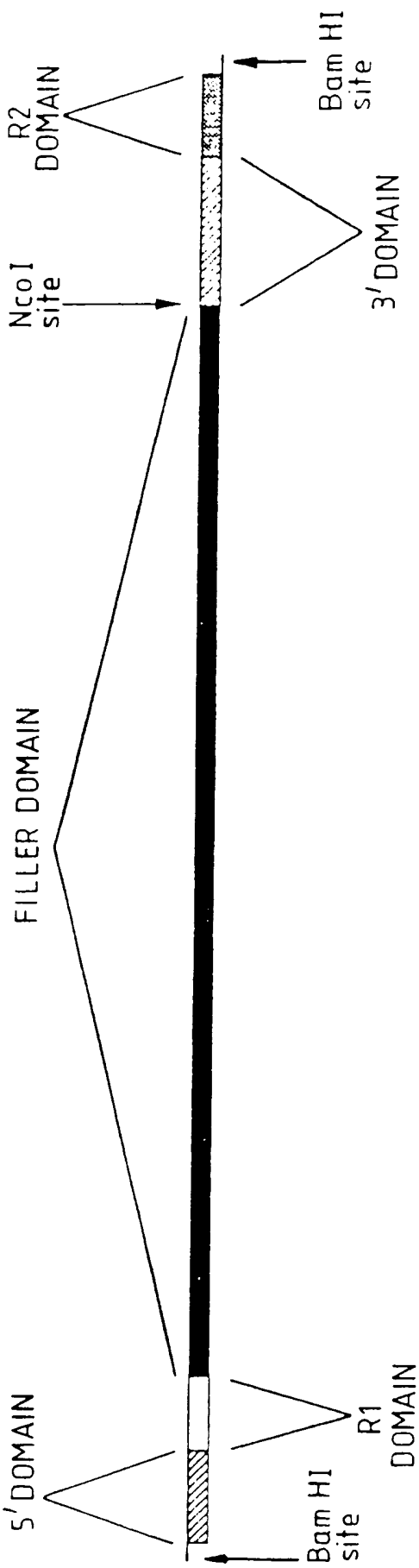
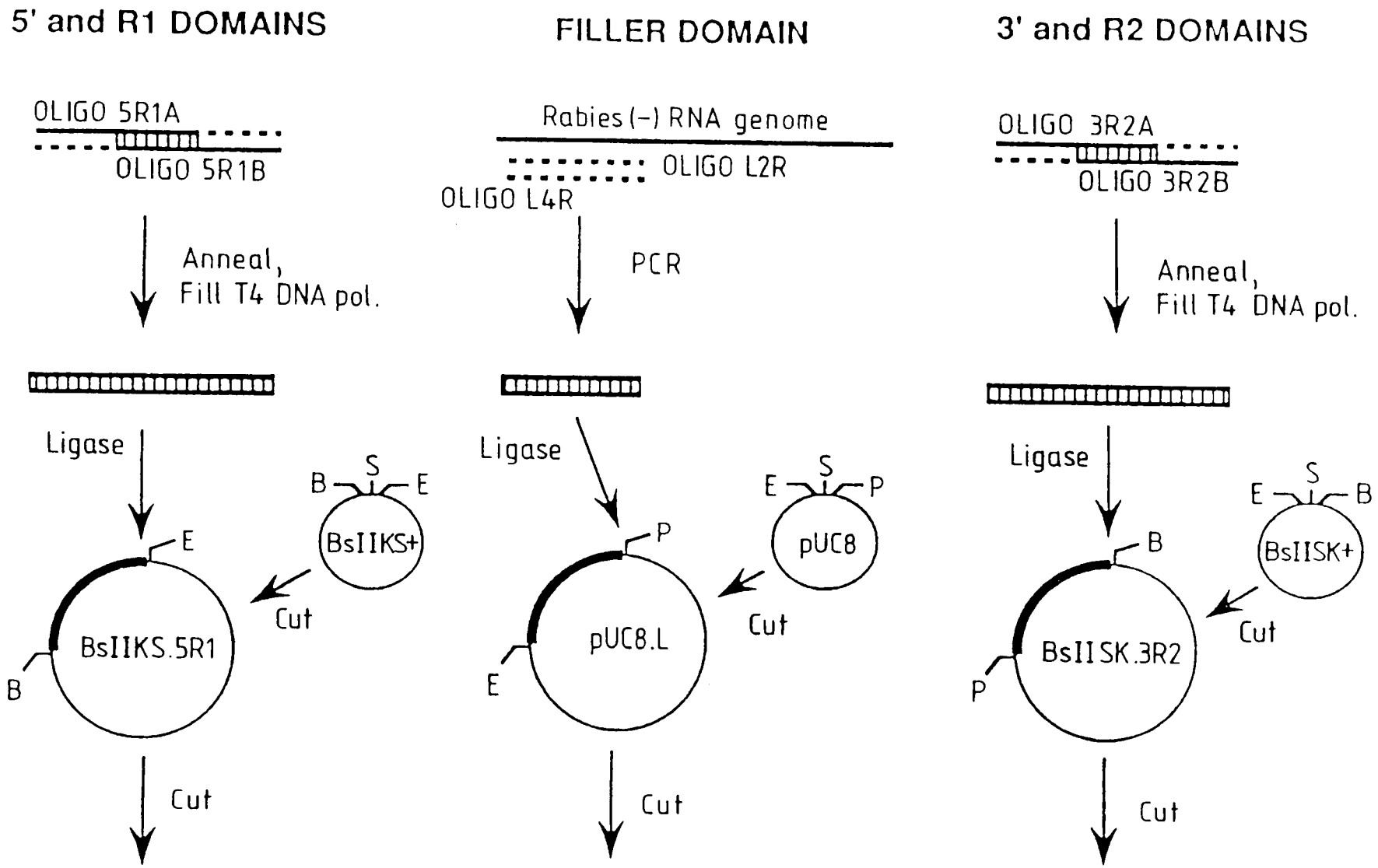


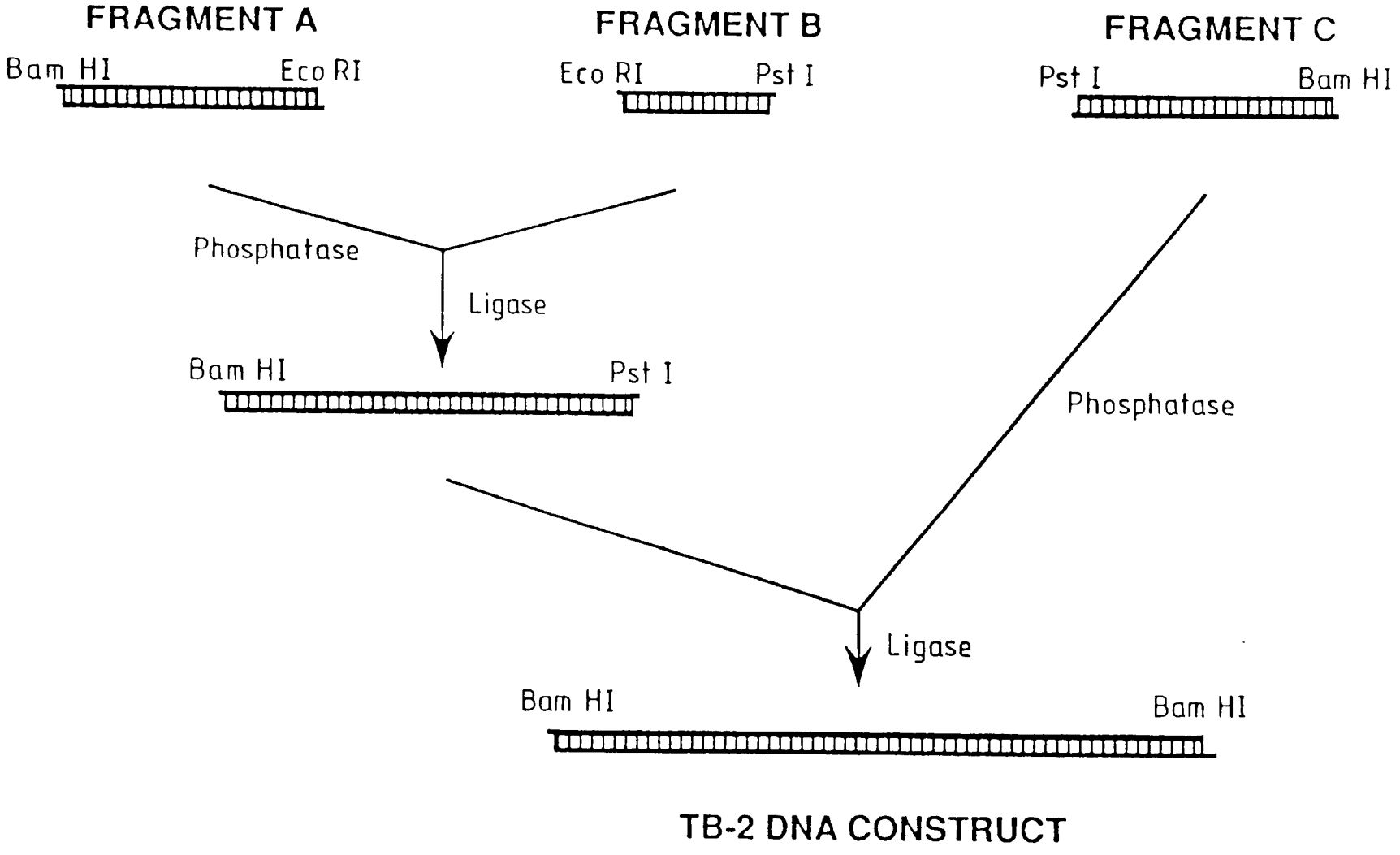
Fig.2.

Fig. 3.



SUBSTITUTE SHEET

Fig.3Cont.



TB-2 DNA CONSTRUCT

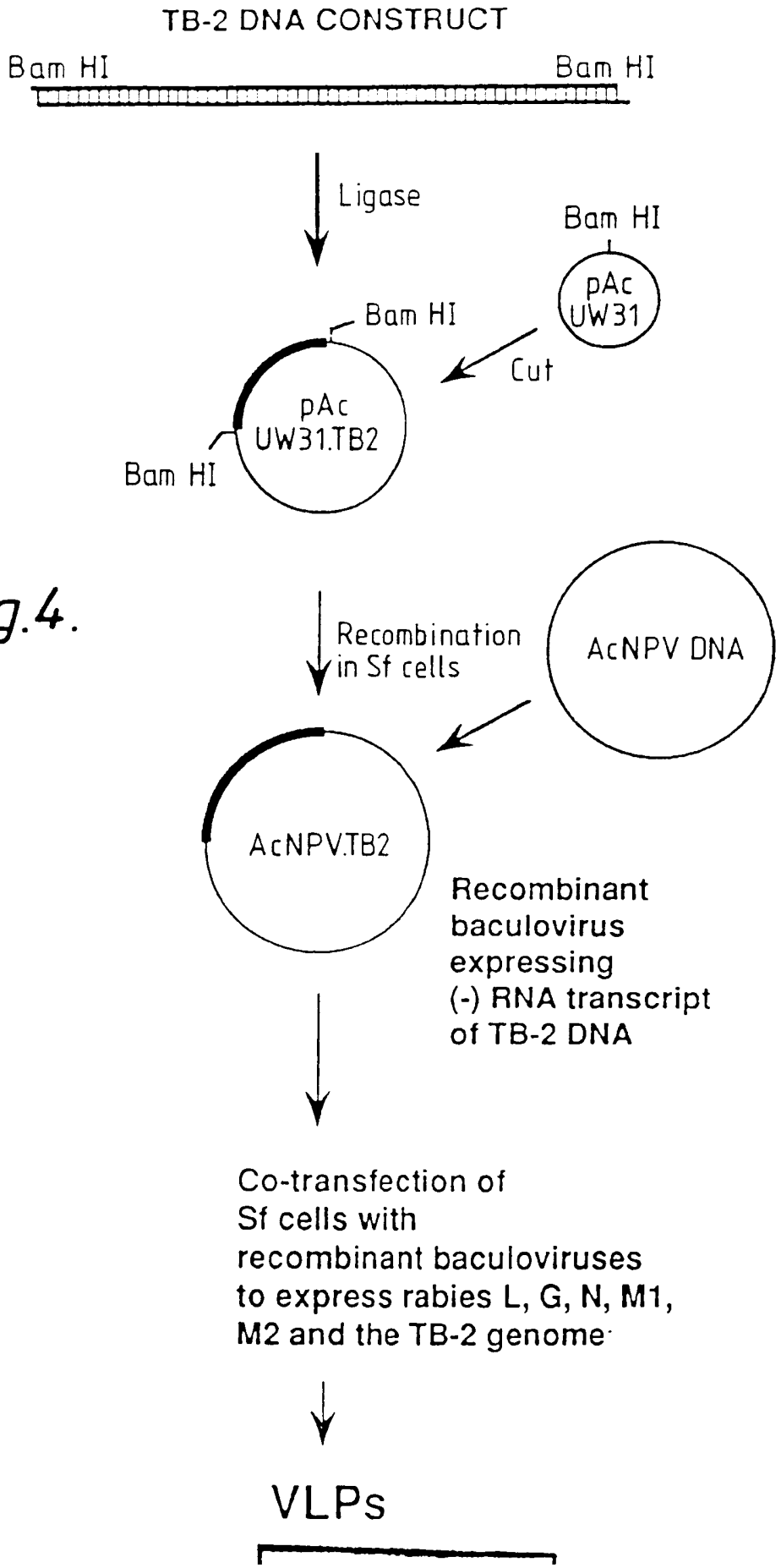

TB-2 DNA CONSTRUCT
Bam HI
Bam HI
Ligase
Bam HI
pAc
UW31
Bam HI
Cut
pAc
UW31.TB2
Bam HI
Recombination
in Sf cells
AcNPV DNA
AcNPV.TB2
Recombinant
baculovirus
expressing
(-) RNA transcript
of TB-2 DNA
Co-transfection of
Sf cells with
recombinant baculoviruses
to express rabies L, G, N, M1,
M2 and the TB-2 genome
VLPs

Fig.4.

Fig.5a.

Plasmid containing anchor G protein gene
(eg rabies G protein gene)

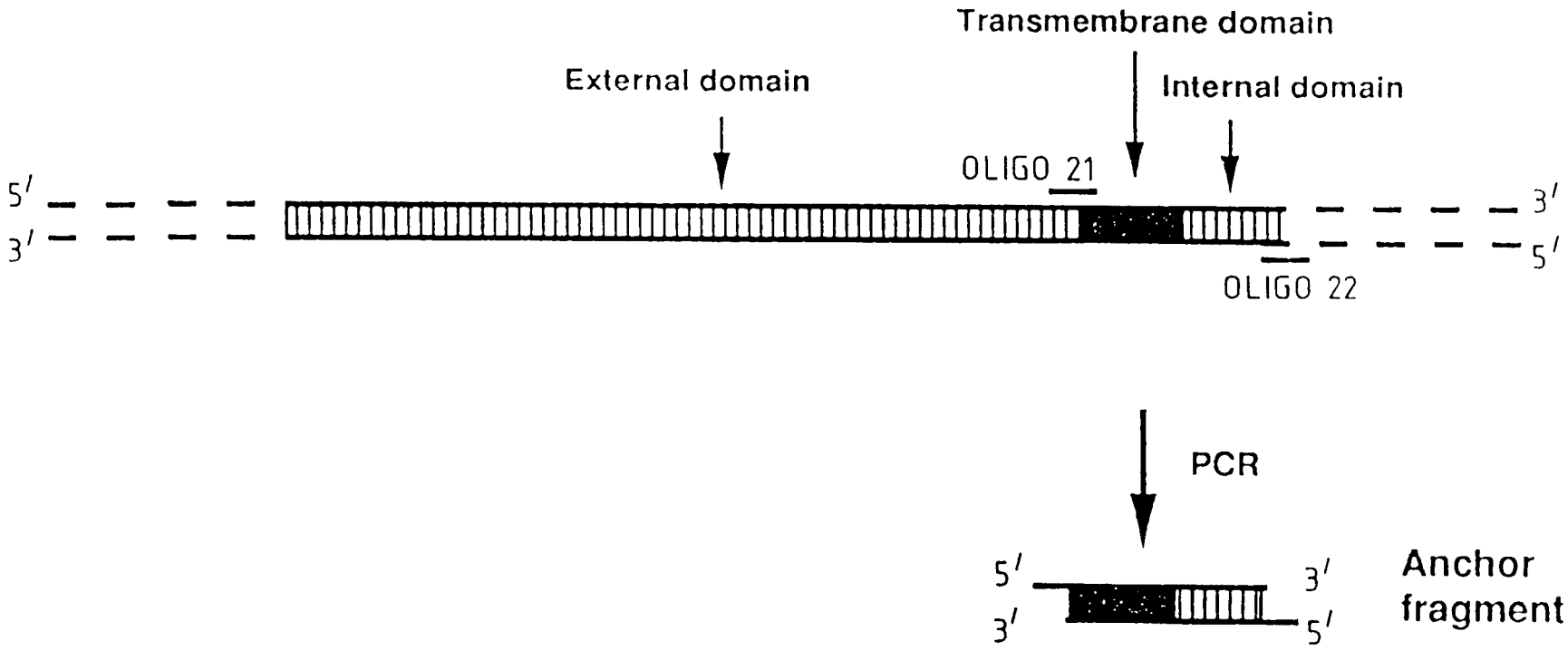


Fig.5b.

Plasmid containing donor G protein gene
(eg VSV G protein gene)

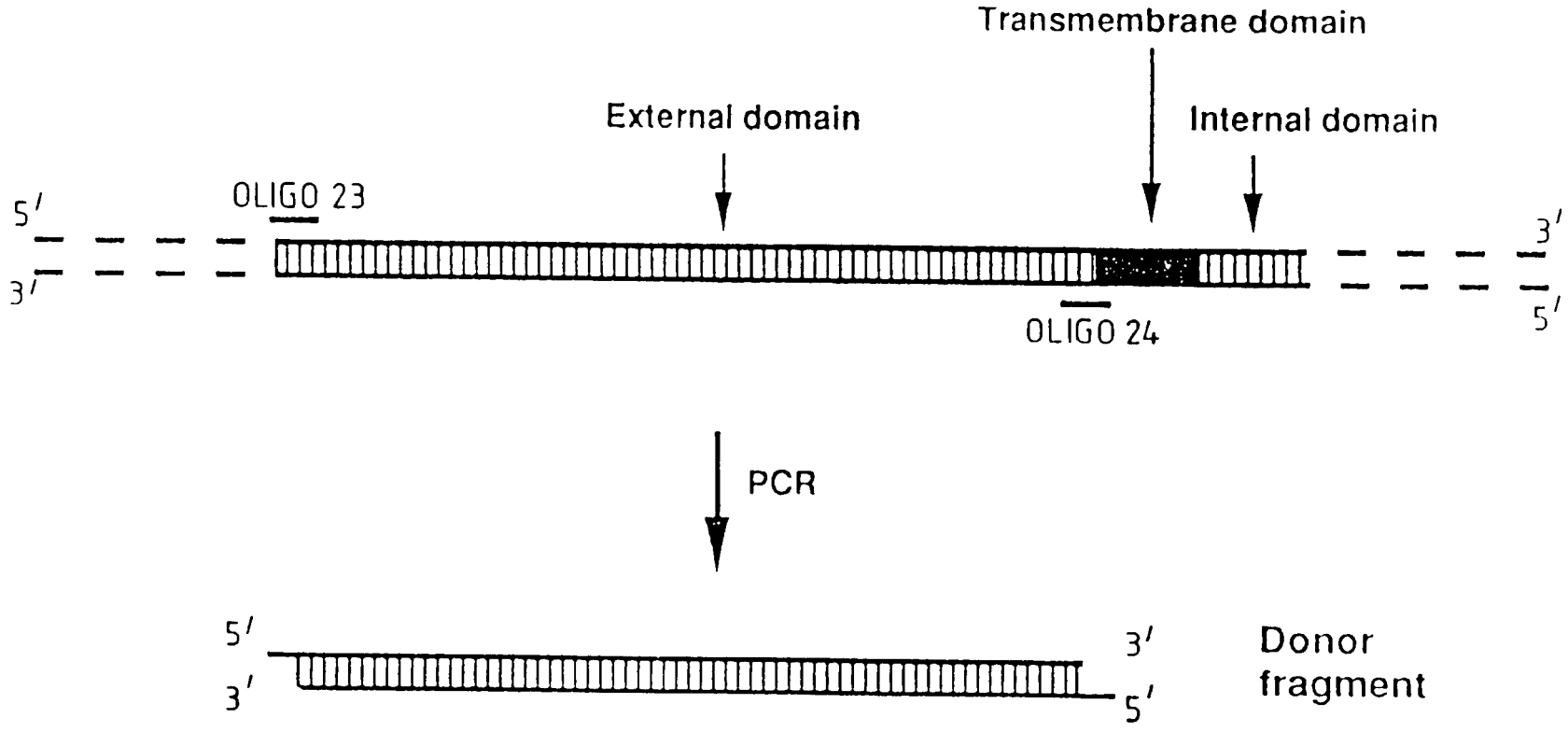
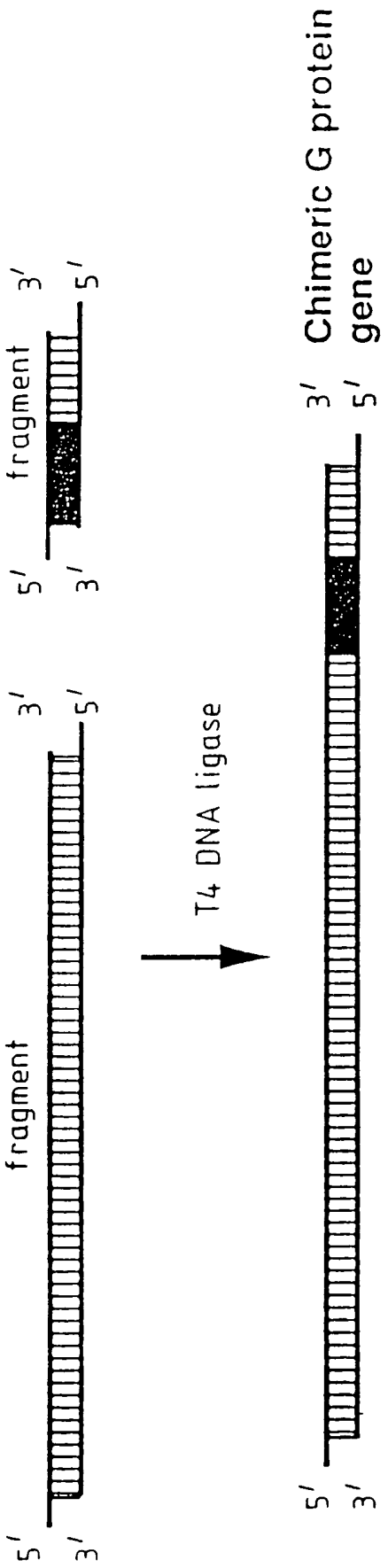


Fig.5c.


Anchor fragment
5′
3′
3′
5′
Donor fragment
3′
5′
5′
3′
T4 DNA ligase
3′
5′
Chimeric G protein gene
5′
3′

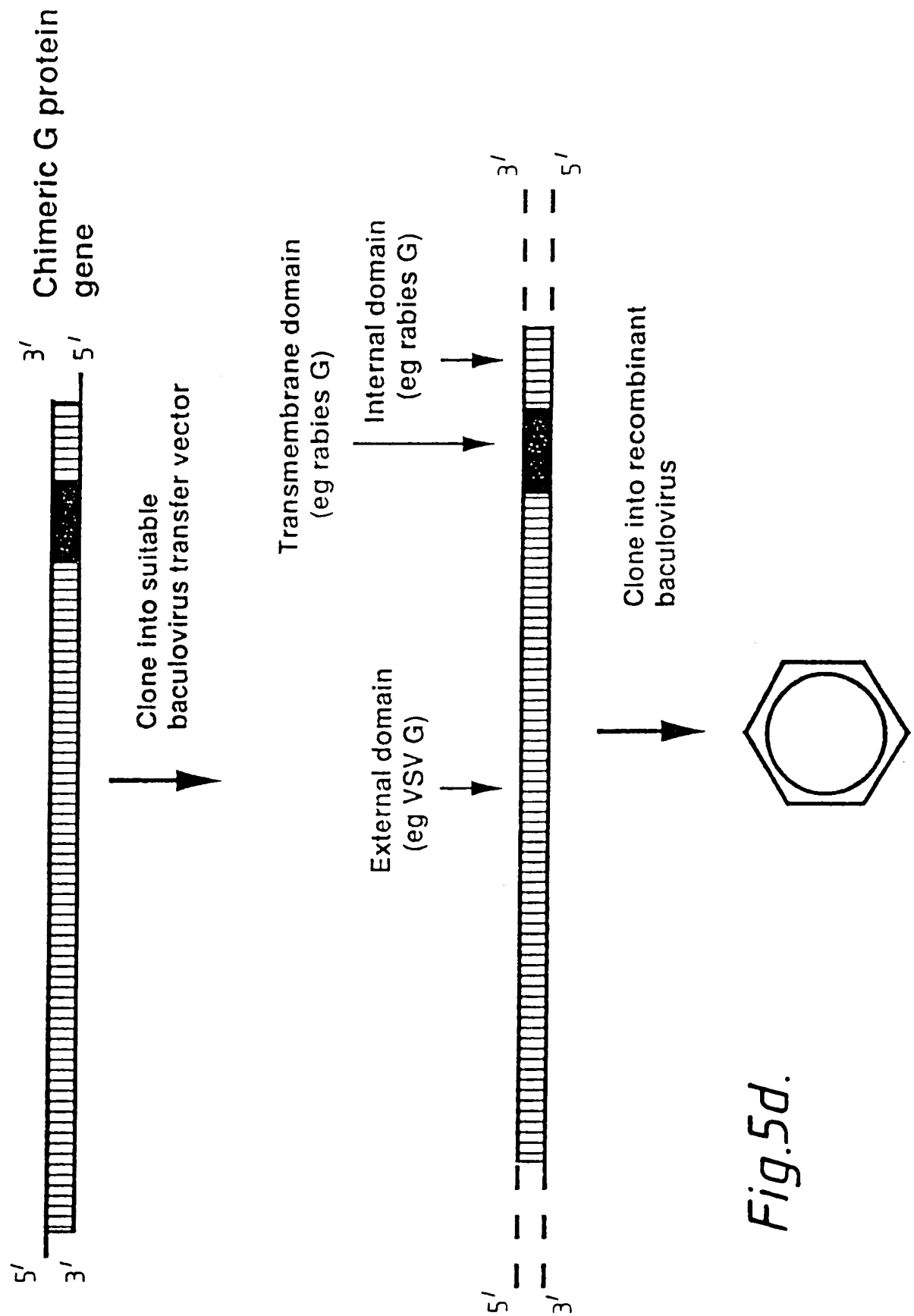

Chimeric G protein gene
3′
5′
Clone into suitable baculovirus transfer vector
Transmembrane domain (eg rabies G)
Internal domain (eg rabies G)
External domain (eg VSV G)
Clone into recombinant baculovirus
5′
3′
3′
5′

Fig.5d.

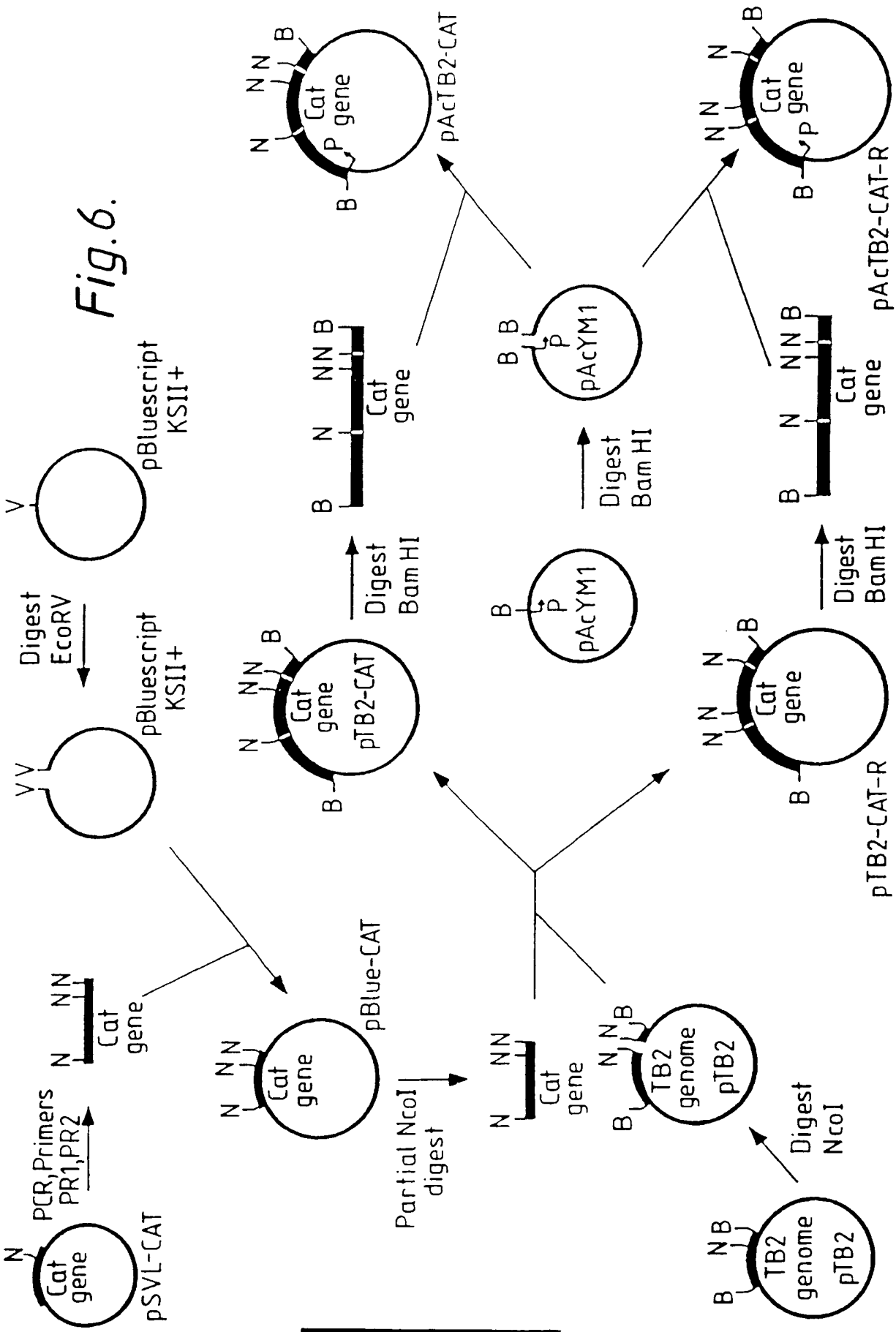

pSVL-CAT
Cat gene
N
PCR, Primers PR1, PR2
N N
Cat gene
N
pBluescript KSII+
V
Digest EcoRV
V V
pBluescript KSII+
pBlue-CAT
Cat gene
N N
N
Partial NcoI digest
N N
Cat gene
N
TB2 genome pTB2
N B
B
Digest NcoI
TB2 genome pTB2
N B
B
pTB2-CAT
Cat gene
N N
N
B
Digest Bam HI
B N NN B
Cat gene
pAcTB2-CAT
Cat gene
N N
N
B
pAcYM1
B
P
pAcYM1
B B
P
Digest Bam HI
B N NN B
Cat gene
pTB2-CAT-R
Cat gene
N N
N
B
Digest Bam HI
N N B
Cat gene
pAcTB2-CAT-R
Cat gene
N N
N
B

Fig. 6.

```
      NcoI
5'-CC ATG GAG AAA AAA ATC ACT GGA TAT ACC ACC GTT GAT ATA TCC CAA   47
     Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr Val Asp Ile Ser Gln
                       5                  10                  15

TGG CAT CGT AAA GAA CAT TTT GAG GCA TTT CAG TCA GTT GCT CAA TGT   95
Trp His Arg Lys Glu His Phe Glu Ala Phe Gln Ser Val Ala Gln Cys
                   20                  25                  30

ACC TAT AAC CAG ACC GTT CAG CTG GAT ATT ACG GCC TTT TTA AAG ACC  143
Thr Tyr Asn Gln Thr Val Gln Leu Asp Ile Thr Ala Phe Leu Lys Thr
               35                  40                  45

GTA AAG AAA AAT AAG CAC AAG TTT TAT CCG GCC TTT ATT CAC ATT CTT  191
Val Lys Lys Asn Lys His Lys Phe Tyr Pro Ala Phe Ile His Ile Leu
           50                  65                  70

GCC CGC CTG ATG AAT GCT CAT CCG GAA TTC CGT ATG GCA ATG AAA GAC  239
Ala Arg Leu Met Asn Ala His Pro Glu Phe Arg Met Ala Met Lys Asp
      75                  80                  85

GGT GAG CTG GTG ATA TGG GAT AGT GTT CAC CCT TGT TAC ACC GTT TTC  287
Gly Glu Leu Val Ile Trp Asp Ser Val His Pro Cys Tyr Thr Val Phe
 90                  95                  100                 105
```

Fig.7a.

```
CAT GAG CAA ACT GAA ACG TTT TCA TCG CTC TGG AGT GAA TAC CAC GAC 335
His Glu Gln Thr Glu Thr Phe Ser Ser Leu Trp Ser Glu Tyr His Asp
                110                 115                 120

                                    T
GAT TTC CGG CAG TTT CTA CAC ATA TAT TCG CAA GAT GTG GCG TGT TAC 383
Asp Phe Arg Gln Phe Leu His Ile Tyr Ser Gln Asp Val Ala Cys Tyr
            125                 Leu 130                 135

GGT GAA AAC CTG GCC TAT TTC CCT AAA GGG TTT ATT GAG AAT ATG TTT 431
Gly Glu Asn Leu Ala Tyr Phe Pro Lys Gly Phe Ile Glu Asn Met Phe
        140                 145                 150

TTC GTC TCA GCC AAT CCC TGG GTG AGT TTC ACC AGT TTT GAT TTA AAC 479
Phe Val Ser Ala Asn Pro Trp Val Ser Phe Thr Ser Phe Asp Leu Asn
    155                 160                 165

                                                NcoI
GTG GCC AAT ATG GAC AAC TTC TTC GCC CCC GTT TTC ACC ATG GGC AAA 527
Val Ala Asn Met Asp Asn Phe Phe Ala Pro Val Phe Thr Met Gly Lys
170                 175                 180                 185

TAT TAT ACG CAA GGC GAC AAG GTG CTG ATG CCG CTG GCG ATT CAG GTT 575
Tyr Tyr Thr Gln Gly Asp Lys Val Leu Met Pro Leu Ala Ile Gln Val
                190                 195                 200
```

Fig.7b.

```
CAT CAT GCC GTT TGT GAT GGC TTC CAT GTC GGC AGA ATG CTT AAT GAA 623
His His Ala Val Cys Asp Gly Phe His Val Gly Arg Met Leu Asn Glu
            205                 210                 215

                                                    NcoI
                                                    CCATGG-3'
TTA CAA CAG TAC TGC GAT GAG TGG CAG GGC GGG GCG TAA CATGAAAAAA  672
Leu Gln Gln Tyr Cys Asp Glu Trp Gln Gly Gly Ala ***
        220                 225                 229

   NcoI
ACCATGG-3' 679
```

Fig.7c.

PCT/AU93/00495

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] C12N 15/64, 15/86, A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
ELECTRONIC DATABASES AS BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC[5]: C12N 15/64, 15/86

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT DATABASES: WPAT, CHEMICAL ABSTRACTS, BIOTECHNOLOGY
KEYWORDS: (SENSE OR ANTISENSE) () RNA(S)(GENOM: OR VIRUS: OR VIRAL OR GENE#) PLUS (AND VECTOR# OR EXPRESS:) IN CHEMICAL ABSTRACTS AND BIOTECHNOLOGY DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	K YAMANKA et al: "In vivo analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA". Proc Natl Acad Sci USA, volume 88, pages 5369-5373, June 1991. See entire document.	1, 2, 8, 9-11, 17, 18 8, 9
Y	W LUYTJES et al: "Amplification, Expression, and Packaging of a Foreign Gene by Influenza Virus" Cell, volume 59, pages 1107-1113, 22 December, 1989. See abstract	1, 8, 9

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 1993 (23.12.93)	13 JAN 1994 (13.01.94)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer Robyn Porter ROBYN PORTER Telephone No. (06) 2832318

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	D E SLEAT et al: "Selective Recovery of foreign gene transcripts as virus-like particles in TMV-infected transgenic tobaccos". Nucleic Acids Research, volume 16, number 8, pages 3127-3140, (1988)	1
A	M J DICKINSON & A PRYOR: "Isometric virus-like particles encapsidate the double-stranded RNA found in Puccinia striiformis, Puccinia recondita, and Puccinia sorghi" Can J Bot, Volume 67, pages 3420-3425, (1989)	
A	M J DICKINSON & A J PRYOR: "Encapsidated and unencapsidated double-stranded RNAs in flax rust, Melampsora lini" Can J Bot, volume 67, pages 1137-1142, 1989	
A	H REVETS et al: "Identification of virus-like particles in Eimeria stiedae". Molecular & Biochemical Parasitology, volume 36, pages 209-216, 1989.	
P,A	AU,A, 23665/92 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 23 February 1993 (23.02.93)	

Box I **Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international search report has not established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically

claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
AU	23665	WO	9301833

END OF ANNEX